

**Amendments to the Specification:**

*Please replace the first paragraph of page 1, pertaining to related applications and introduced by preliminary amendment dated May 3, 1995, with the following amended paragraph:*

This application is a Divisional of U.S. Patent Application Serial application serial No. 07/959,506, filed on October 9, 1992, now U.S. Patent No. 5,500,365, which is a File Wrapper Continuation of U.S. Patent Application Serial application serial No. 07/476,661, filed February 12, 1990, abandoned, which is a Continuation-in-part of U.S. Patent Application Serial application serial No. 07/315,355 filed February 24, 1989, abandoned.

*Please replace the paragraph bridging pages 1 and 2 with the following amended paragraph:*

The potential causes of low steady state levels of mRNA due to the nature of the coding sequence are many. First, full length RNA synthesis might not occur at a high frequency. This could, for example, be caused by the premature termination of RNA during transcription or due to unexpected mRNA processing during transcription. Second, full length RNA could be produced but then processed (splicing, polyA addition) in the nucleus in a fashion that creates a nonfunctional mRNA. If the RNA is properly synthesized, terminated and polyadenylated, it then can move to the cytoplasm for translation. In the cytoplasm, mRNAs have distinct half lives that are determined by their sequences and by the cell type in which they are expressed. Some RNAs are very short-lived and some are much more long-lived. In addtion addition, there is an effect, whose magnitude is uncertain, of translational efficiency on mRNA half-life. In addition, every RNA molecule folds into a particular structure, or perhaps family of sturetures structures, which is determined by its sequence. The particular structure of any RNA might lead to greater or lesser stability in the cytoplasm. Structure per se is probably also a determinant of mRNA processing in the nucleus. Unfortunately, it is impossible to predict, and nearly impossible to determine, the structure of any RNA (except for tRNA) in vitro or in vivo. However, it is likely that dramatically changing the sequence of an RNA will have a large effect on its folded structure. It is likely that structure per se or particular structural features also have a role in determining RNA stability.

*Please replace the paragraph bridging pages 2 and 3 with the following amended paragraph:*

Some particular sequences and signals have been identified in RNAs that have the potential for having a specific effect on RNA stability. This section summarizes what is known about these sequences and signals. These identified sequences often are A+T rich, and thus are more likely to occur in an A+T rich coding sequence such as a *B.t.* gene. The sequence motif ATTTA (or AUUUA as it appears in RNA) has been implicated as a destabilizing sequence in mammalian cell mRNA (Shaw and Kamen, 1986). No analysis of the function of this sequence in plants has been done. Many short lived mRNAs have A+T rich 3' untranslated regions, and these regions often have the ATTTA sequence, sometimes present in ~~multiple~~ multiple copies or as multimers (e.g., ATTTATTTA...). Shaw and Kamen showed that the transfer of the 3' end of an unstable mRNA to a stable RNA (globin or VA1) decreased the stable RNA's half life dramatically. They further showed that a pentamer of ATTTA had a profound destabilizing effect on a stable message, and that this signal could exert its effect whether it was located at the 3' end or within the coding sequence. However, the number of ATTTA sequences and/or the sequence context in which they occur also appear to be important in determining whether they function as destabilizing sequences. Shaw and Kamen showed that a trimer of ATTTA had much less effect than a pentamer on mRNA stability and a dimer or a monomer had no effect on stability (Shaw and Kamen, 1987). Note that multimers of ATTTA such as a pentamer automatically create an A+T rich region. This was shown to be a cytoplasmic effect, not nuclear. In other unstable mRNAs, the ATTTA sequence may be present in only a single copy, but it is often contained in an A+T rich region. From the animal cell data collected to date, it appears that ATTTA at least in some contexts is important in stability, but it is not yet possible to predict which ~~eeurrences~~ occurrences of ATTTA are ~~destabilizing~~ destabilizing elements or whether any of these effects are likely to be seen in plants.

*Please replace the first full paragraph on page 6 with the following amended paragraph:*

In naturally ~~occurring~~ occurring mRNAs that are normally polyadenylated, it has been observed that disruption of this process, either by altering the polyA signal or other sequences in the mRNA, profound effects can be obtained in the level of functional mRNA. This has been observed in several naturally ~~occurring~~ occurring mRNAs, with results that are gene specific so far. There are no general rules that can be derived yet from the study of mutants of these natural genes, and no rules that can be applied to heterologous genes. Below are four examples:

*Please replace the paragraph bridging pages 9 and 10 with the following amended paragraph:*

A plant intron has been moved from a patatin gene into a GUS gene. To do this, site directed mutagenesis was performed to introduce new restriction sites, and this mutagenesis changed several nucleotides in the intron and exon sequences flanking the GT and AG. This intron still functioned properly, indicating the importance of the GT and AG and the flexibility at other nucleotide ~~positons~~ positions. There are of course many ~~occurances~~ occurrences of GT and AG in all genes that do not function as intron splice junctions, so there must be some other sequence or ~~structural~~ structural features that identify splice junctions. In plants, one such feature appears to be base composition per se. Wiebauer et al. (1988) and Goodall et al. (1988) have analyzed plant introns and exons and found that exons have ~50% A+T while introns have ~70% A+T. Goodall et al. (1988) also created an artificial plant intron that has consensus 5' and 3' splice junctions and a random A+T rich internal sequence. This intron was spliced correctly in plants. When the internal segment was replaced by a G+C rich sequence, splicing efficiency was drastically reduced. These two examples ~~demonstrate~~ demonstrate that intron recognition in plants may depend on very general features -- splice junctions that have a great deal of sequence diversity and A+T richness of the intron itself. This, of course, makes it difficult to predict from sequence alone whether any particular sequence is likely to function as an active or partially active intron for RNA processing.

*Please replace the paragraph bridging pages 10-12 with the following amended paragraph:*

As for polyadenylation described above, there are complications in predicting what sequences might be utilized as splice sites in any given gene. First, many naturally ~~occurring~~ occurring genes have alternative splicing pathways that create alternative combinations of exons in the final mRNA (Gallega and Nadal-Ginard, 1988; Helfman and Ricci, 1988; Tsurushita and Korn, 1989). That is, some splice junctions are apparently recognized under some circumstances or in certain cell types, but not in others. The rules governing this are not understood. In addition, there can be an interaction between processing paths such that utilization of a particular polyadenylation site can interfere with splicing at a nearby splice site and vice versa (Adami and Nevins, 1988; Brady and Wold, 1988; Marzluff and Pandey, 1988).

Again no predictive rules are available. Also, sequence changes in a gene can drastically alter the utilization of particular splice junctions. For example, in a bovine growth hormone gene, small deletions in an exon a few hundred bases downstream of an intron cause the splicing efficiency of the intron to drop from greater than 95% to less than 2% (essentially nonfunctional). Other deletions however have essentially no effect (Hampson and Rottman, 1988). Finally, a variety of in vitro and in vivo experiments indicate that mutations that disrupt normal splicing lead to rapid degradation of the RNA in the nucleus. Splicing is a multistep process in the nucleus and mutations in normal splicing can lead to blockades in the process at a variety of steps. Any of these blockades can then lead to an abnormal and unstable RNA. Studies of mutants of normally processed (polyadenylation and splicing) genes are relevant to the study of heterologous genes such as *B.t.* *B.t.* genes might contain functional signals that lead to the production of aberrant nonfunctional mRNAs, and these mRNAs are likely to be unstable. But the *B.t.* genes are perhaps even more likely to contain signals that are analogous to mutant signals in a natural gene. As shown above these mutant signals are very likely to cause defects in the processing pathways whose consequence is to produce unstable mRNAs.

*Please replace the paragraphs covering about page 14, lines 18-28, which are descriptions of Figures 2-4, with the following amended paragraphs:*

Figure 2 illustrates a comparison of the changes in the modified *B.t.k.* HD-1 sequence of Example 1 (lower line (SEQ ID NO:1)) versus the wild-type sequence of *B.t.k.* HD-1 (Cry1Ab) which encodes the crystal protein toxin (upper line (SEQ ID NO:2)).

Figure 3 illustrates a comparison of the changes in the synthetic *B.t.k.* HD-1 sequence of Example 2 (lower line (SEQ ID NO:3)) versus the wild-type sequence of *B.t.k.* HD-1 (Cry1Ab) which encodes the crystal protein toxin (upper line (SEQ ID NO:4)).

Figure 4 illustrates a comparison of the changes in the synthetic *B.t.k.* HD-73 (Cry1Ac/Cry1Ab hybrid) sequence of Example 3 (lower line (SEQ ID NO:5)) versus the wild-type sequence of *B.t.k.* HD-73 (upper line (SEQ ID NO:6)).

*Please replace the paragraphs spanning about page 15, line 7, to page 16, line 8, which are descriptions of Figures 8-14, with the following amended paragraphs:*

Figure 8 illustrates a comparison of the changes in the synthetic truncated *B.t.k.* HD-73 gene (Amino acids 29-615 with an N-terminal Met-Ala) of Example 3 (lower line (SEQ ID NO:7)) versus the wild-type sequence of *B.t.k.* HD-73 (upper line (SEQ ID NO:8)).

Figure 9 illustrates a comparison of the changes in the synthetic/wild-type full length *B.t.k.* HD-73 sequence of Example 3 (lower line (SEQ ID NO:9)) versus the wild-type full-length sequence of *B.t.k.* HD-73 (upper line (SEQ ID NO:10)).

Figure 10 illustrates a comparison of the changes in the synthetic/modified full length *B.t.k.* HD-73 sequence of Example 3 (lower line (SEQ ID NO:11)) versus the wild-type full-length sequence of *B.t.k.* HD-73 (upper line (SEQ ID NO:10)).

Figure 11 illustrates a comparison of the changes in the fully synthetic full-length *B.t.k.* HD-73 sequence of Example 3 (lower line (SEQ ID NO:12)) versus the wild-type full-length sequence of *B.t.k.* HD-73 (upper line (SEQ ID NO:10)).

Figure 12 illustrates a comparison of the changes in the synthetic *B.t.t.* sequence of Example 5 (lower line (SEQ ID NO:14)) versus the wild-type sequence of *B.t.t.* which encodes the crystal protein toxin (Cry3Aa) (upper line (SEQ ID NO:15)).

Figure 13 illustrates a comparison of the changes in the synthetic *B.t.* P2 sequence of Example 6 (lower line (SEQ ID NO:16)) versus the wild-type sequence of *B.t.k.* HD-1 which encodes the P2 protein toxin (Cry2Aa) (upper line (SEQ ID NO:17)).

Figure 14 illustrates a comparison of the changes in the synthetic *B.t. entomocidus* sequence of Example 7 (lower line (SEQ ID NO:18)) versus the wild-type sequence of *B.t. entomocidus* which encodes the Btent protein toxin (Cry1Ca) (upper line (SEQ ID NO:19)).

*Please replace the paragraph beginning at about page 16, line 11, which is a description of Figure 16, with the following amended paragraph:*

Figure 16 illustrates a comparison of the changes in the synthetic potato leaf roll virus (PLRV) coat protein sequence of Example 9 (lower line (SEQ ID NO:20)) versus the wild-type coat protein sequence of PLRV (upper line (SEQ ID NO:21)).

*Please replace the paragraph bridging pages 21 – 22 with the following amended paragraph:*

Due to the degeneracy of the genetic code and the limited number of codon choices for any amino acid, most of the "excess" A+T of the structural coding sequences of some *Bacillus* species are found in the third position of the codons. That is, genes of some *Bacillus* species have A or T as the third nucleotide in many codons. Thus A+T content in part can determine codon usage bias. In addition, it is clear that genes evolve for maximum function in the organism in which they evolve. This means that particular nucleotide sequences found in a gene from one organism, where they may play no role except to code for a particular stretch of amino acids, have the potential to be recognized as gene control elements in another organism (such as transcriptional promoters or terminators, polyA addition sites, intron splice sites, or specific mRNA degradation signals). It is perhaps surprising that such misread signals are not a more common feature of heterologous gene expression, but this can be explained in part by the relatively homogeneous A+T content (~50%) of many organisms. This A+T content plus the nature of the genetic code put clear constraints on the ~~likelihood~~ likelihood of occurrence of any particular oligonucleotide sequence. Thus, a gene from *E. coli* with a 50% A+T content is much less likely to contain any particular A+T rich segment than a gene from *B. thuringiensis*.

*Please replace the text in Table III from page 37 at line 12 through line 30 with the following:*

BTK185	18	TCCCCAGATA ATATCAAC
	<u>(SEQ ID NO:22)</u>	
BTK240	48	GGCTTGATTC CTAGCGAACT
	<u>(SEQ ID NO:23)</u>	CTTCGATTCT CTGGTTGATG
		AGCTGTTC
BTK462	54	CAAAAATGAG AGGTGGAGGT
	<u>(SEQ ID NO:24)</u>	TGGCAGCTTG AACGTACACG
		GAGAGGAGAGGAAC
BTK669	48	AGTTAGTGTA AGCTCTCTTC
	<u>(SEQ ID NO:25)</u>	TGAACTGGTT GTACCTGATC
		CAATCTCT
BTK930	39	AGCCATGATC TGGTGACCGG
	<u>(SEQ ID NO:26)</u>	ACCAGTAGTA TTCTCCTCT
BTK1110	32	AGTTGTTGGT TGTTGATCCC
	<u>(SEQ ID NO:27)</u>	GATGTTAAAA GG

*Please replace the text in Table III from page 38 at line 8 through line 18 with the following:*

BTK1380A	37	GTGATGAAGG GATGATGTTG
	<u>(SEQ ID NO:28)</u>	TTGAACTCAG CACTACG
BTK1380T	100	CAGAACGTTCC AGAGCCAAGA
	<u>(SEQ ID NO:29)</u>	TTAGTAGACT TGGTGAGTGG
		GATTTGGGTG ATTTGTGATG
		AAGGGATGAT GTTGTGAAC
		TCAGCACTAC GATGTATCCA
BTK1600	27	TGATGTGTGG AACTGAAGGT
	<u>(SEQ ID NO:30)</u>	TTGTGGT

*Please replace the paragraph bridging pages 38-39 with the following:*

The regions for mutagenesis were selected in the following manner. All regions of the DNA sequence of the *B.t.k.* gene were identified which contained five or more consecutive base pairs which were A or T. These were ranked in terms of length and highest percentage of A+T in the surrounding sequence over a 20-30 base pair region. The DNA was then analysed analyzed for regions which might contain polyadenylation sites (see Table II above) or ATTAA sequences. Oligonucleotides were designed which maximized the elimination of A+T consecutive regions which contained one or more polyadenylation sites or ATTAA sequences. Two potential plant polyadenylation sites were rated more critical (see Table II) based on published reports. Codons were selected which increased G+C content, did not generate restriction sites for enzymes useful for cloning and assembly of the modified gene (BamHI, BglIII, SacI, NcoI, EcoRV) and did not contain the doublets TA or GC which have been reported to be infrequently found in codons in plants. The oligonucleotides were at least 18 bp long ranging up to 100 base pairs and contained at least 5-8 base pairs of direct homology to native sequences at the ends of the fragments for efficient hybridization and priming in site-directed mutagenesis reactions. Figure 2 compares the wild-type *B.t.k.* HD-1 gene sequence (cry1Ab; SEQ ID NO:2) with the sequence which resulted from the modifications by site-directed mutagenesis (SEQ ID NO:1).

*Please replace the consecutive paragraphs starting on page 40 at line 5 through page 42 line 5 with the corresponding consecutive paragraphs as follows:*

BTK185 (SEQ ID NO:22) is an 18-mer used to eliminate a plant polyadenylation site in the midst of a nine base pair region of A+T.

BTK240 (SEQ ID NO:23) is a 48-mer. Seven base pairs were changed by this oligonucleotide to eliminate three potential polyadenylation sites (2 AACCAA, 1 AATTAA). Another region close to the region altered by BTK240, starting at bp 312, had a high A+T content (13 of 15 base pairs) and an ATTAA region. However, it did not contain a potential polyadenylation site and its longest string of uninterrupted A+T was seven base pairs.

BTK462 (SEQ ID NO:24) is a 54-mer introducing 13 base pair changes. The first six changes were to reduce the A+T richness of the gene by replacing wild-type codons with codons

containing G and C while avoiding the CG doublet. The next seven changes made by BTK462 were used to eliminate an A+T rich region (13 of 14 base pairs were A or T) containing two ATTTA regions.

BTK669 (SEQ ID NO:25) is a 48-mer making nine individual base pair changes eliminating three possible polyadenylation sites (ATATAA, AATCAA, and AATTAA) and a single ATTTA site.

BTK930 (SEQ ID NO:26) is a 39-mer designed to increase the G+C content and to eliminate a potential polyadenylation site (AATAAT - a major site). This region did contain a nine base pair region of consecutive A+T sequence. One of the base pair changes was a G to A because a G at this position would have created a G+C rich region (CCGG(G)C). Since sequencing reactions indicate that there can be difficulties generating sequence through G+C consecutive bases, it was thought to be prudent to avoid generating potentially problematic regions even if they were problematic only in vitro.

BTK1110 (SEQ ID NO:27) is a 32-mer designed to introduce five changes in the wild-type gene. One potential site (AATAAT - a major site) was eliminated in the midst of an A+T rich region (19 of 22 base pairs).

BTK1380A (SEQ ID NO:28) and BTK1380T (SEQ ID NO:29) are responsible for 14 individual base pair changes. The first region (1380A) has 17 consecutive A+T base pairs. In this region is an ATTTA and a potential polyadenylation site (AATAAT). The 100-mer (1380T) contains all the changes dictated by 1380A. The large size of this primer was in part an experiment to determine if it was feasible to utilize large oligonucleotides for mutagenesis (over 60 bases in length). A second consideration was that the 100-mer was used to mutagenize a template which had previously been mutageneized mutagenized by 1380A. The original primer ordered to mutagenize the region downstream and adjacent to 1380A did not anneal efficiently to the desired site as indicated by an inability to obtain clean sequence utilizing the primer. The large region of homology of 1380T did assure proper annealing. The extended size of 1380T was more of a convenience rather than a necessity. The second region adjacent to 1380A covered by 1380T has a high A+T content (22 of 29 bases are A or T).

BTK1600 (SEQ ID NO:30) is a 27-mer responsible for five individual base pair changes. An ATTTA region and a plant polyadenylation site were identified and the appropriate changes engineered.

*Please replace the paragraph bridging pages 42 and 43 with the following amended paragraph:*

Referring to Table IV modified *B.t.k.* HD-1 genes were constructed that contained all of the above modifications (pMON5370) or various subsets of individual modifications. These genes were inserted into pMON893 for plant transformation and tobacco plants containing these genes were analyzed. The analysis of tobacco plants with the individual modifications was undertaken for several reasons. Expression of the wild type truncated gene in tobacco is very poor, resulting in infrequent identification of plants toxic to THW. Toxicity is defined by leaf feeding assays as at least 60% mortality of tobacco hornworm neonate larvae with a damage rating of 1 or less (scale is 0 to 4; 0 is equivalent to total protection, 4 total damage). The modified HD-1 gene (pMON5370) shows a large increase in expression (estimated to be approximately 100-fold; see Table VIII) in tobacco. Therefore, increases in expression of the wild-type gene due to individual individual modifications would be apparently a large increase in the frequency of toxic tobacco plants and the presence of detectable *B.t.k.* protein. Results are shown in the following table:

*Please replace the paragraph at about page 52, lines 8-30, which comprises the first portion of the contents of Table VI, with the following amended paragraph:*

73K1363	51	AATACTATCG GATGCGATGA
<u>(SEQ ID NO:31)</u>		TGTTGTTGAA CTCAGCACTA
		CGGTGTATCC A
73K1437	33	TCCTGAAATG ACAGAACCGT
<u>(SEQ ID NO:32)</u>		TGAAGAGAAA GTT
73K1471	48	ATTTCACACTG CTGTTGAGTC
<u>(SEQ ID NO:33)</u>		TAACGAGGTC TCCACCAGTG
		AATCCTGG
73K1561	[ [60] ] 61	GTGAATAGGG GTCACAGAAG
<u>(SEQ ID NO:34)</u>		CATACCTCAC ACGAACTCTA
		TATCTGGTAG <u>ATGTTGGATGG</u>
		<u>ATGTTGGATG G</u>
73K1642	33	TGTAGCTGGA ACTGTATTGG
<u>(SEQ ID NO:35)</u>		AGAAGATGGA TGA
73K1675	48	TTCAAAGTAA CCGAAATCGC
<u>(SEQ ID NO:36)</u>		TGGATTGGAG ATTATCCAAG
		GAGGTAGC
73K1741	39	ACTAAAGTTT CTAACACCCA
<u>(SEQ ID NO:37)</u>		CGATGTTACC GAGTGAAGA

*Please replace the paragraph at page 53, lines 8-13, which comprises the second portion of the contents of Table VI, with the following amended paragraph:*

73K1797 <u>(SEQ ID NO:38)</u>	36	AACTGGAATG AACTCGAATC TGTCGATAAT CACTCC
73KTERM <u>(SEQ ID NO:39)</u>	54	GGACACTAGA TCTTAGTGAT AATCGGTAC ATTTGTCTTG AGTCCAAGCT GGTT

*Please replace the paragraph bridging pages 67 and 68 with the following amended paragraph:*

The full length *B.t.k.* HD-73 genes described in Example 3 were also incorporated into the plant transformation vector pMON893 so that they were expressed from the En 35S promoter. The synthetic/wild-type full length HD-73 gene of Figure 9 was incorporated into pMON893 to create pMON10505 pMON10506. The synthetic/modified full length HD-73 gene of Figure 10 was incorporated into pMON893 to create pMON10526. The fully synthetic HD-73 gene of Figure 11 was incorporated into pMON893 to create pMON10518. These vectors were used to obtain transformed tobacco plants, and the plants were analyzed for insecticidal efficacy and for *B.t.k.* HD-73 protein levels by Western blot or ELISA immunoassay.

*Please replace the paragraph bridging pages 78-79 with the following amended paragraph:*

The generality of the increased expression of *B.t.k.* HD-1 and *B.t.k.* HD-73 by use of the modified and synthetic genes was extended to cotton. Transgenic calli were produced which contain the wild type (pMON9921) and the synthetic HD-1 (pMON5377) genes. Here again the *B.t.k.* HD-1 protein produced from calli containing the wild-type gene was not detected, whereas calli containing the synthetic HD-1 gene expressed the HD-1 protein at easily detectable levels. The HD-1 protein was produced at approximately 1000 ng/mg of plant calli extract protein.

Again, to ensure that the protein produced by the transgenic cotton calli was biologically active and that the increased expression observed with the synthetic gene translated to increased biological activity, extracts of cotton calli were made in similar manner as described for tobacco plants, except that the calli was first dried between Whatman filter paper to remove as much of the water as possible. The dried calli were then ground in liquid nitrogen and ground in 100 mM sodium carbonate buffer, pH 10. Approximately 0.5 ml aliquotes aliquots of this material was applied to tomato leaves with a paint brush. After the leaf dried, five tobacco hornworm larvae were applied to each of two leaf samples. Leaves painted with extract from control calli were completely destroyed. Leaves painted with extract from calli containing the wild-type HD-1 gene (pMON9921) showed severe damage. Leaves painted with extract from calli containing the synthetic HD-1 gene (pMON5377) showed no damage (see Table XIV below).

*Please replace the paragraph bridging pages 84-85 with the following amended paragraph:*

Sterile shoot cultures of Russet Burbank are maintained in vials containing 10 ml of PM medium (Murashige and Skoog (MS) inorganic salts, 30 g/l ~~sucrose~~ sucrose, 0.17 g/l NaH<sub>2</sub>PO<sub>4</sub>H<sub>2</sub>O, 0.4 mg/l thiamine-HC1, and 100 mg/l myo-inositol, solidified with 1 g/l Gelrite at pH 6.0). When shoots reached approximately 5 cm in length, stem internode segments of 7-10 mm are excised and smeared at the cut ends with a disarmed *Agrobacterium tumefaciens* vector containing the synthetic *B.t.t.* gene from a four day old plate culture. The stem explants are co-cultured for three days at 23Ec on a sterile filter paper placed over 1.5 ml of a tobacco cell feeder layer overlaid on 1/10 P medium (1/10 strength MS inorganic salts and organic addenda without casein as in Jarret et al. (1980), 30 g/l ~~sucrose~~ sucrose and 8.0 g/l agar). Following co-culture the explants are transferred to full strength P-1 medium for callus induction, composed of MS inorganic salts, organic additions as in Jarret et al. (1980) with the exception of casein, 3.0 mg/l benzyladenine (BA), and 0.01 mg/l naphthaleneacetic acid (NAA) (Jarret, et al., 1980). Carbenicillin (500 mg/l) is included to inhibit bacterial growth, and 100 mg/l kanamycin is added to select for transformed cells. After four weeks the explants are transferred to medium of the same composition but with 0.3 mg/l gibberellic acid (GA3) replacing the BA and NAA (Jarret et al., 1981) to promote shoot formation. Shoots begin to develop approximately two weeks after transfer to shoot induction medium; these are excised and transferred to vials of PM medium for rooting. Shoots are tested for kanamycin resistance conferred by the enzyme neomycin phosphotransferase II, by placing a section of the stem onto callus induction medium

containing MS organic and inorganic salts, 30 g/l sucrose, 2.25 mg/l BA, 0.186 mg/l NAA, 10 mg/l GA3 (Webb, et al., 1983) and 200 mg/l kanamycin to select for transformed cells.

*Please replace the paragraph on page 89 from line 6 through line 20 with the following amended paragraph:*

The genes encoding the P2 protein although distinct in sequence from the *B.t.k.* HD-1 and HD-73 genes share many common features with these genes. In particular, the P2 protein genes have a high A+T content (65%), multiple potential polyadenylation signal sequences (26) and numerous ATTAA sequences (10). Because of its overall similarity to the poorly expressed wild-type *B.t.k.* HD-1 and HD-73 genes, the same problems are expected in expression of the wild-type P2 gene as were encountered with the previous examples. Based on the above-described method for designing the synthetic *B.t.* genes, a synthetic P2 gene has been designed which gene should be expressed at adequate levels for protection in plants. A ~~comparision~~ comparison of the wild-type and synthetic P2 genes is shown in Figure 13.

*Please replace the paragraph on page 91 from line 5 through line 24 with the following amended paragraph:*

The genes encoding the Btent protein although distinct in sequence from the *B.t.k.* HD-1 and HD-73 genes share many common features with these genes. In particular, the Btent protein genes have a high A+T content (62%), multiple potential polyadenylation signal sequences (39 in the full length coding sequence and 27 in the first 1875 nucleotides that is likely to encode the active toxic fragment) and numerous ATTAA sequences (16 in the full length coding sequence and 12 in the first 1875 nucleotides). Because of its overall similarity to the poorly expressed wild type *B.t.k.* HD-1 and HD-73 genes, the wild-type Btent genes are expected to exhibit similar problems in expression as were encountered with the wild-type HD-1 and HD-73 genes. Based on the above-described method used for designing the other synthetic *B.t.* genes, a synthetic Btent gene has been designed which gene should be expressed at adequate levels for protection in plants. A ~~comparision~~ comparison of the wild type and synthetic Btent genes is shown in Figure 14.

*Please replace the paragraph bridging pages 95-96 with the following amended paragraph:*

The leaves were incubated at room temperature for 24 hours. The pMON752 samples were stained with a substrate that allows visual detection of the GUS gene product. This analysis showed that over one hundred spots in each sample were expressing the GUS product and the [[the]] triplicate samples showed very similar levels of GUS expression. For the pMON744 and pMON8643 samples 5 larvae of tobacco hornworm were added to each leaf and allowed to feed for 48 hours. All three samples bombarded with pMON744 showed extensive feeding damage and no larval mortality. All three samples bombarded with pMON8643 showed no evidence of feeding damage and 100% larval mortality. The samples were also assayed for the presence of *B.t.k.* protein by a qualitative immunoassay. All of the pMON8643 samples had detectable *B.t.k.* protein. These results demonstrated that the [[the]] synthetic *B.t.k.* gene was expressed in differentiated corn plant tissue at insecticidal levels.

*Please replace the first full paragraph on page 98 from lines 1 through 19 with the following amended paragraph:*

The original PLRV sequence contains two potential plant polyadenylation signals (AACCAA and AAGCAT) and both of [[the]] these occur in the 3' noncoding sequence that has been removed in the synthetic gene. The original PLRV gene also contains [[on]] one ATTAA sequence. This is also contained in the 3' noncoding sequence, and is in the midst of the longest stretch of uninterrupted A+T in the gene (a stretch of 7 A+T nucleotides). This sequence was removed in the synthetic gene. Thus, sequences that the algorithm of Figure 1 targets for change have been changed in the synthetic PLRV coat protein gene by removal of the 3' noncoding segment. Within the coding sequence, codon changes were also made to remove three other regions of sequence described above. In particular, two regions of 5 consecutive A+T and one region of 5 consecutive G+C within the coding sequence have been removed in the synthetic gene.

*Please replace the paragraph bridging pages 101-102 with the following amended paragraph:*

When SSU promoters were used in combination with their CTP, the DNA fragments extended through the coding sequence of the CTP and a small portion of the mature SSU coding sequence at which point an NcoI restriction site was engineered by standard techniques to allow the in frame fusion of *B.t.* coding sequences with the CTP. In particular, for the petunia SSU11a CTP, *B.t.* coding sequences were fused to the SSU sequence after amino acid 8 of the mature SSU sequence at which point the NcoI site was placed. The 8 amino acids of mature SSU sequence were included because preliminary in vitro chloroplast uptake experiments indicated that uptake was of *B.t.k.* was observed only if this segment of mature SSU was included. For the Arabidopsis *ats1A* CTP, the complete CTP was included plus 24 amino acids of mature SSU sequence plus the sequence gly-gly-arg-val-asn-cys-met-gln-ala-met (SEQ ID NO:40), terminating in an NcoI site for *B.t.* fusion. This short sequence reiterates the native SSU CTP cleavage site (between the cys and met) plus a short segment surrounding the cleavage site. This sequence was included in order to insure proper uptake into chloroplasts. *B.t.* coding sequences were fused to this *ats1A* CTP after the met codon. In vitro uptake experiments with this CTP construction and other (non-*B.t.*) coding sequences showed that this CTP did target proteins to the chloroplast.

*Please replace the paragraph at page 103, lines 5-9, with the following amended paragraph:*

A variety of plant transformation vectors were constructed utilizing either the truncated synthetic [[.]] HD-73 coding sequence of Figure 4 or the full length *B.t.k.* HD-73 coding sequence of Figure 11. These are listed in the table below.

*Please replace the last full paragraph on page 105 from line 19 through line 30 with the following amended paragraph:*

Tobacco plants containing the full length synthetic HD-73 fused to the SSU11A CTP and driven by the En 35S promoter produced levels of *B.t.k.* protein and insecticidal activity comparable to pMON1518 pMON10815 which contains does not include the CTP. In addition,

for pMON10518 pMON10815 the *B.t.k.* protein extracted from plants was observed by gel electrophoresis to contain multiple forms less than full length, apparently due the cleavage of the C-terminal portion (not required for toxicity) in the cytoplasm. For pMON10814, the majority of the protein appeared to be intact full length indicating that the protein has been stabilized from proteolysis by targeting to the chloroplast.

*Please replace the paragraph bridging pages 106-107 with the following amended paragraph:*

In plants as well as other eucaryotes, proteins that are destined to be localized either extracellularly or in several specific compartments are typically synthesized with an N-terminal amino acid extension known as the signal peptide. This signal peptide directs the protein to enter the compartmentalization pathway, and it is typically cleaved from the mature protein as an early step in compartmentalization. For an extracellular protein, the secretory pathway typically involves cotranslational insertion into the endoplasmic reticulum with cleavage of the signal peptide ~~occurring~~ occurring at this stage. The mature protein then passes thru the Golgi body into vesicles that fuse with the plasma membrane thus releasing the protein into the extracellular space. Proteins destined for other compartments follow a similar pathway. For example, proteins that are destined for the endoplasmic reticulum or the Golgi body follow this scheme, but they are specifically retained in the appropriate compartment. In plants, some proteins are also targeted to the vacuole, another membrane bound compartment in the ~~cytoplasm~~ cytoplasm of many plant cells. Vacuole targeted proteins diverge from the above pathway at the Golgi body where they enter vesicles that fuse with the vacuole.

*Please replace the paragraph bridging pages 107-108 with the following amended paragraph:*

A common feature of this protein targeting is the signal peptide that initiates the compartmentalization process. Fusing a signal peptide to a protein will in many cases lead to the targeting of that protein to the endoplasmic reticulum. The efficiency of this step may depend on the sequence of the mature protein itself as well. The signals that direct a protein to a specific compartment rather than to the extracellular space are not as clearly defined. It appears that many of the signals that direct the protein to specific compartments are contained within the amino acid sequence of the mature protein. This has been shown for some vacuole targeted

proteins, but it is not yet possible to define these sequences precisely. It appears that secretion into the extracellular space is the "default" pathway for a protein that contains a signal sequence but no other compartmentalization signals. Thus, a strategy to direct *B.t.* proteins out of the cytoplasm is to fuse the genes for synthetic *B.t.* genes to DNA sequences encoding known plant signal peptides. These fusion genes will give rise to *B.t.* proteins that enter the secretory pathway, and lead to extracellular extracellular secretion or targeting to the vacuole or other compartments.

*Please replace the paragraph bridging pages 108-109 with the following amended paragraph:*

Signal sequences for several plant genes have been described. One such sequence is for the tobacco pathogenesis related protein PR1b described by Cornelissen et al. The PR1b protein is normally localized to the extracellular space. Another type of signal peptide is contained on seed storage proteins of legumes. These proteins are localized to the protein body of seeds, which is a vacuole like compartment found in seeds. A signal peptide DNA sequence for the beta subunit of the 7S storage protein of common bean (*Phaseolus vulgaris*), PvB has been described by Doyle et al. Based on the published these published sequences, genes were synthesized by chemical synthesis of oligonucleotides that encoded the signal peptides for PR1b and PvB. The synthetic genes for these signal peptides corresponded exactly to the reported DNA sequences. Just upstream of the translational initiation initiation codon of each signal peptide a BamHI and BglII site were inserted with the BamHI site at the 5' end. This allowed the insertion of the signal peptide encoding segments into the BglII site of pMON893 for expression from the En 35S promoter. In some cases to achieve secretion or compartmentalization of heterologous proteins, it has proved necessary to include some amino acid sequence beyond the normal cleavage site of the signal peptide. This may be necessary to insure proper cleavage of the signal peptide. For PR1b the synthetic DNA sequence also included the first 10 amino acids of mature PR1b. For PvB the synthetic DNA sequence included the first 13 amino acids of mature PvB. Both synthetic signal peptide encoding segments ended with NcoI sites to allow fusion in frame to the methionine initiation codon of the synthetic *B.t.* genes.